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GRANT NUMBER DAMD17-96-1-6138

TITLE: Recombinant Vaccine Strategies for Breast Cancer Prevention

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REPORT DATE: October 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1998	Annual (1 Oct 97 - 30 Sep 98)

4. TITLE AND SUBTITLE
Recombinant Vaccine Strategies for Breast Cancer Prevention

5. FUNDING NUMBERS
DAMD17-96-1-6138

6. AUTHOR(S)
Elizabeth M. Jaffee, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Johns Hopkins University
Baltimore, MD 21205-5014

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Exciting new findings in autoimmune disease and cancer have led to the realization that a large set of antigenic determinants of the self have not induced self-tolerance. These peptide determinants could provide target structures for autoimmune attack as well as antitumor immune responses. We hypothesized that vaccine strategies can be devised that specifically generate an immune response against breast ductal epithelial cells. Since the overwhelming majority of breast tumors arise in these cells, destroying these cells prior to the development of neoplasia will effectively prevent cancer. We are attempting to augment the immune response to the breast-specific antigen, HER-2/neu, which is expressed by mammary tissue in HER-2/neu transgenic mice prior to mammary tumor development, by enhancing the T cell response using selected vectors that may alter antigen processing, thereby influencing antigen-specific immunity. We have evaluated vaccinia constructs that express antigen alone or together with the cytokine GM-CSF, to determine if this immunity can be further enhanced. In our preliminary studies, we have found that this vaccinia approach is superior to vaccination with plasmid DNA either intramuscularly or via gene gun delivery. We will next compare this vaccinia approach with vaccinia carrying the HER2/neu gene along with the lysosomal targeting molecule, LAMP-1. This study will provide a paradigm for novel vaccine approaches to breast cancer prevention.

14. SUBJECT TERMS Breast Cancer
Vaccines, gene therapy, immunotherapy, tissue specific antigens, HER-2/neu, tumor immunology

15. NUMBER OF PAGES 23

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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E Scott Jeffee 10/6/98

PI - Signature Date

Recombinant Vaccine Strategies for Breast Cancer Prevention

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5. INTRODUCTION.

Breast cancer is a common problem, with an incidence of 182,000 new cases a year, and a mortality rate of 46,000 deaths per year. Overall, women in the United States have a 1 in 9 lifetime incidence of developing breast cancer (1-3). Because of its high incidence, breast cancer is a significant public health problem. Currently, most research is aimed at treating this disease. The time has come to focus efforts on prevention.

Recently, two breast cancer genes, BRCA1 and BRCA2, have been identified which influences susceptibility to breast cancer (4-6). Other genes associated with breast cancer susceptibility are likely to be identified in the future. It is estimated that approximately 5% of all patients who develop breast cancer, and 25% of patients who are diagnosed with breast cancer before age 30, will have a demonstrated susceptibility (7). With the current advances in molecular technology and the intensified effort to sequence the entire human genome, it is also very likely that other breast cancer susceptibility genes will be identified in the near future that are expressed by women at higher risk for breast cancer who do not have a strong family history. The identification of susceptibility genes should allow genetic screening for predisposition to this common deadly disease. Currently, the only option for prevention is surgical removal of the breast, which results in significant dysfiguration and psychological trauma.

The goal of cancer prevention is to develop treatment modalities which specifically target the breast ductile tree, as well as preneoplasia and incipient tumor cells. Vaccine strategies that result in T cell activation directed against self proteins expressed by these cells have the potential to be effective treatment for this purpose because the diversity of the T cell repertoire allows for the recognition of greater than 10^6 distinct peptide determinants. In addition, it is well-established that T cells recognize peptide fragments of cellular proteins bound to major histocompatibility molecules (MHC) on the surface of cells, and any cellular protein can be presented to T cells in this way.

Recent data have demonstrated that many antigenic determinants of the self do not induce self tolerance (8-9). Instead, these peptide determinants can become antigenic targets for autoimmune attack, and therefore, may be potential targets for directing antitumor immune responses. Strong support of this concept comes from data demonstrating that the majority of human melanoma antigens that have been identified are normal tissue peptides that are overexpressed by the melanoma (10-16). Most importantly, melanoma patients receiving *in vitro* expanded tumor infiltrating lymphocytes (TIL) specific for a few of these antigens demonstrated clinical responses against metastases without evidence of significant tissue damage (17-18). Interestingly, *in vitro* cytotoxic T cells (CTL) can be generated against these same antigens from peripheral blood lymphocytes (PBL) of healthy donors without a history of autoimmunity (19). These data are consistent with the

large body of evidence that both a critical density of MHC-peptide complexes on the target cell surface, as well as a critical set of co-stimulatory molecules, are required to activate T cells. As these signals continue to be defined, it should be possible to manipulate them toward beneficial immune responses that result in preventative treatments, without causing unwanted toxicity.

Our recent studies have provided strong evidence that the antitumor response can be considerably enhanced by recruiting subsets of lymphocytes that respond to tumor-specific antigens. Using murine models, we have demonstrated that the injection of tumor cells genetically engineered to produce local concentrations of cytokines, results in the activation of tumor-specific T cells capable of generating systemic antitumor immunity (20-22). In one study that compared over 10 cytokines for their ability to augment antitumor immunity, tumor cells genetically-altered to secrete granulocyte-macrophage colony stimulating factor (GM-CSF) produced the most potent systemic anti-tumor immunity (22). Analysis of the immune response generated has revealed that systemic immunity is dependent on both $CD4^+$ and $CD8^+$ T cells. Vaccination with GM-CSF secreting tumor cells has been shown to immunize mice against subsequent injections of parental tumor, as well as cure mice burdened with a relatively small amount of pre-established tumor. In addition, we have recently completed a phase I trial evaluating this approach in patients with advanced renal cell carcinoma (23). The results of this trial are extremely promising in that they demonstrate both clinical and immunologic activity. In addition, 3/3 patients receiving the bioactive dose of GM-CSF secreting vaccine cells demonstrated a DTH reaction against both autologous tumor cells, and autologous normal renal cells, yet no functional evidence of autoimmune damage was observed. This study provides further support for the existence of tissue-specific antigens that can serve as immune rejection targets in a second human tumor.

Two scientific questions need to be addressed prior to the design of vaccines that can prevent the development of breast cancer. The first requirement is the identification of antigenic targets expressed early by the pre-malignant cell, that can be used for immunization. The second requirement is the identification of optimal methods for delivering these antigens to the immune system for priming antitissue immune responses.

Currently, the Her2/neu gene product, which is overexpressed by 25-40% of invasive malignancies (24), and by 56% of pure ductal carcinoma in situ (DCIS) (25), is the best antigen for targeting immune responses to prevent the development of breast cancer. There are two reasons for this. First, HER2/neu is selectively overexpressed by premalignant and malignant cells, but is also expressed by normal breast ductal tissue (26). Second, HER2/neu encodes a large protein (1255 amino acids) and therefore should contain a range of epitopes capable of binding to different MHC alleles. One major concern is that patients would be immunologically tolerant to self-proteins like HER2/neu and that immunity might be difficult to generate. However,

Disis and colleagues recently described the identification of HER2/neu specific antibodies in 11 of 20 breast cancer patients studied. Interestingly, none of these patients had evidence of autoimmunity (27). A second study demonstrated $CD8^+$ T cell responses against HER2/neu peptides from lymphocytes of a normal donor (28). These studies demonstrate the existence of T cell precursors that have the potential to recognize the endogenous HER2/neu gene product.

Muller and colleagues have developed a transgenic mouse that overexpresses the proto-oncogene Her2/neu under the MMTV promoter (29). The mice develop focal mammary tumors at approximately 6-8 months of age that metastasize with high frequency to lung. Expression of the proto-oncogene product in histopathologically normal mammary epithelium has been demonstrated prior to the development of these focal mammary tumors. There are at least two reasons why this model provides a unique opportunity to study antigen-based vaccine strategies for the prevention of naturally developing mammary tumors. 1) Because the Her2/neu product is overexpressed in the normal ductal epithelium of these mice prior to the development of mammary tumors, this model provides a unique opportunity to evaluate vaccine strategies for preventing the development of spontaneously arising primary tumors. 2) the product of the proto-oncogene HER2/neu is an excellent target for evaluating recombinant vaccine strategies for augmenting antigen-specific immunity, since it is overexpressed in several common human tumors.

The HER2/neu transgenic mouse model is being used to directly compare antigen-based vaccine strategies for the prevention of the development of mammary tumors. Specifically, we are evaluating vectors that express antigen alone or together with co-stimulatory molecules or cytokines to determine if this immunity can be further enhanced. The vectors that are being tested include: 1) plasmid vectors delivered intramuscularly or intradermally using a gene gun; 2) Vaccinia vectors carrying the HER-2/neu antigen alone, or a fusion protein consisting of HER-2/neu and the lysosomal targeting molecule (LAMP-1); 3) Listeria monocytogenes vectors. All three vaccine approaches were chosen based on previous experience demonstrating that these vectors can augment potent specific antitumor immunity against existing cancers (30-33).

One major concern with employing antigen-based vaccine approaches that target rejection antigens expressed by normal tissue prior to the development of malignancy, is that these antigens may go unrecognized by activated T cells at the levels at which they are expressed. Our collaborator, Dr. Sara Sukumar, has developed a gene therapy approach which can selectively ablate mammary epithelial cells in a rat model of breast cancer. Using viral vectors with high efficiency of infection (in particular, vaccinia and adenovirus vectors), her group has demonstrated the feasibility of preferentially targeting the epithelial cells by directly injecting the vectors into the primary mammary duct through the teat. Our working hypothesis is that

we can increase access of activated T cells to normal ductal epithelial antigens by locally infusing vectors carrying a suicide gene such as the Herpes thymidine kinase (HTK) into the primary mammary duct, thereby rendering them susceptible to the toxic lethal effects of an antiviral drug such as gancyclovir (34). Therefore, we are also testing the synergistic effects of combining this unique approach of accessing mammary epithelium with our recombinant vaccine strategies.

6. BODY

A. Hypothesis being tested (Assumptions).

This proposal is testing the hypothesis that the generation of autoimmunity against breast epithelial cell-specific antigens using recombinant vaccines will result in the destruction of the ductile tree, preneoplasias, and incipient tumor cells, thereby preventing breast cancer. The hypothesis is based on the fact a significant number of human melanoma antigens that are the targets of T cells have recently been identified, and the majority of these antigens are normal tissue-specific antigens. These antigens are expressed by 40-60% of other patient's tumors, which provides strong support for the existence of common sets of antigens that can serve as targets for antigen-specific vaccine strategies. Most importantly, these antigens have served as tumor rejection antigens *in vivo*, resulting in clinical responses without functional evidence of destructive autoimmunity.

B. Experimental Methods.

Using the HER2/neu proto-oncogene transgenic mouse model of breast cancer, and the product of HER2/neu as a tissue-specific antigen for targeting immune responses, we are testing three recombinant vaccine strategies for the ability to: 1) activate antigen-specific immune responses and 2) prevent the development of breast cancer. These strategies are first being optimized in the parental FVB/N mouse in which tolerance against the HER-2/neu mouse is not expected. Once the vaccine strategy can prevent the development of mammary tumors in the parental line, we then test the optimized vaccine approach in the HER-2/neu transgenic mouse which demonstrates peripheral tolerance against the HER-2/neu gene product. To accomplish this goal, we are conducting the following experiments.

1. Generation and testing of Recombinant DNA plasmid vectors.

Summary of findings from the first year of funding.

During the first year of this proposal, we tested two different naked DNA plasmid vectors for immune priming in the parental FVB/N mice. The first plasmid, pSvneo, expressed the HER-2/neu gene under the SV40 promoter. We performed intramuscular injections and compared the HER-2/neu expressing plasmid DNA alone with HER-2/neu expressing plasmid

DNA combined with the murine GM-CSF expressing plasmid DNA for protection against subsequent challenge of the NT2 tumor. We did not demonstrate any protection against challenge with the NT2 mammary tumor lines that derived from spontaneously developing tumors in the HER-2/neu transgenic mice. Although we detected good expression of the neu gene in vitro, we were concerned that the SV40 promoter was not being expressed well *in vivo*. In addition, we were concerned that this vector did not contain the bacterial immune stimulating sequences recently reported to be required for optimal immune priming (35). We therefore cloned the neu gene into the pcDNA3 vector (invitrogen) under the CMV promoter, which allows for expression under the strong and most universal CMV promoter. We constructed and tested three plasmids: a plasmid containing the entire HER-2/neu gene; a plasmid containing the murine GM-CSF gene; and a plasmid containing the murine B71 co-stimulatory molecule. In addition, we already had available the pcDNA3 plasmid containing the control antigen influenza A hemagglutinin (HA) which is also a transmembrane protein similar to the product of the HER-2/neu gene. All three plasmids were confirmed to be functional by transfection into COS cells. In our first set of experiments which we reported in the first year of funding, we performed intramuscular injections and compared the HER-2/neu expressing plasmid DNA alone with HER-2/neu expressing plasmid DNA combined with the murine GM-CSF expressing plasmid DNA for protection against subsequent challenge of the NT5 tumor. We have now generated five mammary tumor lines from endogenously developing mammary tumors in the HER-2/neu transgenic mice. All of these lines express equivalent levels of neu protein. We have chosen to use NT5 for all of our *in vivo* studies because it is the most aggressive tumor. As a negative control we compared these mice with mice that received the influenza A HA gene mixed with the murine GM-CSF plasmid. In the FVB/N mice, thirty percent of the mice receiving the HER-2/neu and GM-CSF expressing plasmids combined were tumor free at 55 days following vaccination. FVB/N mice receiving either the HER-2/neu plasmid vector alone or the control HA vector plus murine GM-CSF vector developed tumors by 50 days following challenge, although there seemed to be a significant delay in tumor development in the mice receiving the HER-2/neu plasmid alone. All of the HER-2/neu transgenic mice succumbed to tumor by day 30 following challenge, regardless of whether they received the HER-2/neu plasmid alone, the HER-2/neu plasmid mixed with the GM-CSF plasmid, or the HA plasmid mixed with the GM-CSF plasmid.

We believe that these studies required further optimization since only a small effect was seen with the HER-2/neu plasmid combined with the GM-CSF plasmid in the FVB/N parental mice which we are using as our control for determining the effectiveness of our vaccine strategies in immunocompetent mice that have not been exposed to the immunizing antigen.

The cell type that is being transfected *in vivo* with intramuscular injections of DNA is unknown. If muscle cells are the cells expressing the gene delivered by plasmid DNA then a significant $CD4^+$ T cell response would not be expected. Yet, $CD4^+$ T cells have been determined to be critical for immune response amplification and memory. It is possible that professional antigen presenting cells (APCs) are being transfected with the DNA or are taking up antigen from the muscle cells by some yet unknown mechanism, but this hypothesis has not been proven. Injection of DNA plasmids with a gene gun may directly deliver the DNA to professional APCs such as langerhans cells that reside at the interface of the epidermis and dermis (which is the greatest delivery depth that the gene gun can achieve). This would allow activation of a $CD4^+$ and $CD8^+$ T cell response and therefore enhance the potency of this approach.

We tested the gene gun approach in the second set of experiments. Mice receiving the vaccination by gene gun were compared with mice receiving the vaccine by intramuscular injection as described above for the first set of experiments. 100% of mice receiving vaccination by intramuscular injection succumbed to tumor by 70 days post challenge with NT5 mammary tumor cells. In contrast, 50% of mice receiving the plasmid DNA by gene gun were tumor free 70 days post-challenge. These studies imply that delivery by the gene gun may be a more efficient way of activating an antitumor immune response in the HER-2/neu mice. However, we still believed that it would be beneficial to be able to improve the potency of the gene gun delivery by optimizing delivery conditions in the FVB/N mice since, as discussed in the first year report, the HER-2/neu transgenic mice demonstrate peripheral tolerance to the HER-2/neu antigen.

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At the conclusion of year one (10/97), we planned to optimize conditions for vaccination with naked DNA plasmids in FVB/N mice using the gene gun. We planned to evaluate the following parameters: 1) dose of DNA; 2) number of boost injections; 3) the spatial distribution of simultaneous injections among 3 or more lymph node regions (36); 4) HER-2/neu antigen given together with the co-stimulatory molecule B7-1, B7-2, GM-CSF, and with the lysosomal targeting molecule LAMP-1.

a. Procedure.

To optimize immunization with gene gun delivery of naked DNA, a set of experiments were performed to compare immunization with either: a single intradermal 1 ug injection of the DNA vaccine given once; a single intradermal 1 ug injection of the DNA vaccine given twice, each one month apart; three 1 ug injections of DNA vaccine given simultaneously at three different lymph node regions. 10 mice per group received either: 1) the HER-2/neu gene mixed with the pcDNA3 wildtype vector; 2) the HER-2/neu gene

mixed with the GM-CSF gene; 3) the HER-2/neu gene mixed with the B7.1 gene; 4) the HER-2/neu gene mixed with the B7.2 gene; 5) the HA gene mixed with the GM-CSF gene as a negative control; 6) no vaccination. Mice were challenged 2 weeks after the last vaccination with $5x10^6$ (parental mice) and $5x10^4$ (transgenic mice) NT5 mammary tumor cells.

b. Results.

After repeating several experiments, we have found that a single dose of vaccine at a single site is just as potent at generating anti-HER-2/neu specific immunity when compared with either two vaccinations 2 weeks apart, or the spatial distribution of the vaccinating plasmid. The results of one experiment that is representative of our optimized vaccine is demonstrated in **figure 1**. 100% of the FVB/N mice (**figure 1A**) receiving the HER-2/neu plus GM-CSF DNA vaccine remained tumor free for more than 60 days before this cohort began developing tumors. This is in contrast to mice receiving no vaccine or the HA plus GM-CSF control DNA vaccine. 100% of mice in these two control groups developed tumors by day 28 following inoculation with tumor cells. In addition, the HER-2/neu plus GM-CSF combination appeared to be more potent than combination of the HER-2/neu plasmid with either B7.1 or B7.2 co-stimulatory genes. Unfortunately, none of these vaccines were potent enough to prevent tumor development in the transgenic mice (**figure 1B**).

c. Discussion.

These studies imply that delivery by the gene gun is potent enough to significantly delay tumor development in the FVB/N mice. We do not believe that the eventual development of tumors is a result of a less than longer express HER-2/neu surface protein the tumors that grow out no longer express HER-2/neu surface protein nor are they recognized by HER-2/neu specific T cells. *In vitro* growth of explanted tumor cells regain surface HER-2/neu expression after one week in culture and are subsequently detected and lysed by HER-2/neu specific T cells. To confirm that the FVB/N mice are still adequately immunized against the HER-2/neu protein at the time the original tumor develops, we rechallenged all vaccinated mice with $5x10^6$ HER-2/neu expressing tumors on the opposite limb to which the original tumor was given on day 35 following the original inoculation with tumor. Normally, vaccinated mice begin to develop tumors starting on day 40. As demonstrated in **figure 2**, all of the mice that were vaccinated are developing tumors at the original site of vaccination. However, none of these mice have developed tumor at the rechallenge site in the opposite limb. Thus, we believe, that we have optimized this approach adequately. We will have to evaluate other approaches for prevention of tumor development in the transgenic mice.

d. Future Studies.

During the next funding period we will attempt to improve the potency of the naked DNA vaccine in the transgenic mice by evaluating synergy of this approach with the vaccinia vector approach described below (37). In addition, we will evaluate whether manipulation of the initial immune response with antibody to CTLA4, a co-stimulatory molecule on T cells that may play a critical role in pushing the T cell response toward tolerance (38), can enhance the potency of the DNA vaccine approach.

e. Recommendations in relation to the Statement of Work.

We expected to complete optimization studies testing the DNA vectors by the end of year two. We have accomplished this. We have identified a gene gun delivery approach as being superior to the intramuscular injections as well as to our gold standard vaccine approach (autologous tumor cells genetically modified to express the cytokine GM-CSF). In year three, we will be comparing this vaccine approach with other vectors as well as with the addition of systemic modulators of the immune response.

2. Generation of Recombinant Vaccinia vectors.

Progress Report for the funding period 9/97 to 10/98 .

a/b. Procudures and Results.

At the end of year one we were in the process of constructing several recombinant vaccinia vectors with and without the lysosomal targeting molecule (LAMP-1) for testing in our mouse model. We initially had difficulty constructing a vaccinia vector containing the HER-2/neu gene. In particular, we cut the entire rat HER-2/neu gene out of the psv2/neu vector obtained from William Muller who originally developed the HER-2/neu transgenic mice. However, one recurrent problem that we faced was that all of the restriction sites that could be used to accomplish this could not eliminate the inclusion of a 3' untranslated region. Although this was adequate for constructing a vaccinia vector containing the HER-2/neu gene alone, we could not use this HER-2/neu gene to create the bicistronic constructs (containing HER-2/neu and the LAMP-1 molecule). We then attempted to amplify the gene using PCR but due to the large size of the gene (4Kb) repeated attempts resulted in approximately 2 to 3 mutations/500 base pairs. We have therefore decided to use the original HER-2/neu gene which was excised from the psv2/neu vector. We successfully cloned the entire rat HER-2/neu cDNA into the pSC11 cloning vector for recombination into wildtype vaccinia virus. We then cloned the HER-2/neu gene into the vaccinia vector. We have now created the bicistronic construct containing the LAMP-1 targeting molecule and the HER-2/neu gene by performing a second recombination step. In addition to producing recombinant vaccinia which expresses the entire HER-2/neu gene product, we have made a series of constructs consisting of the putative HER-2/neu membrane insertion signal

sequence followed by one of ten overlapping 400 base pair segments of the HER-2/neu gene with and without fusion to the LAMP-1 transmembrane and cytoplasmic domain in order to identify portions of this large gene that encodes for the relevant antigenic peptides. So far we have completed cloning of each 400 bp segment with and without LAMP-1 into the pSC11 vector. We have confirmed that we have the correct size DNA expected for each clone. Some of these fragments have been cloned into vaccinia and are in the process of being tested for expression. All of these fragments have been cloned into the pcDNA3 plasmid and have been transfected into NIH3T3 cells. We are now picking transfected clones. Transfected clones will first be tested for integration of the HER-2/neu segment by PCR. If present, we will then test the clones for recognition by our HER-2/neu specific T cells.

c. Discussion.

The vaccinia vectors containing the entire HER-2/neu gene with and without LAMP-1 that have been constructed are now undergoing testing *in vivo* and the results of these preliminary tests are discussed below in the next section. These vectors containing minigenes should allow us to identify smaller portions of the gene for insertion into non-vaccinia vectors that do not easily allow cloning of 4 Kb genes. In addition, these vectors should allow us to more easily identify MHC class I and II restricted antigenic peptides for the H-2q background for more specific immune monitoring of the *in vivo* studies.

d. Future Studies.

We are now confirming the sequence and function of the bicistronic HER-2/neu/LAMP-1 construct that has been cloned into vaccinia. In addition, we are currently cloning the HER-2/neu minigenes into the vaccinia vector. We will then test each vector for *in vitro* expression. Fluorescent microscopy to localize the HER-2/neu gene in the lysosomal compartment will be performed to confirm in vitro expression of the HER-2/neu/LAMP-1 construct. We will infect NIH3T3 cells with the vaccinia constructs containing the HER-2/neu minigenes. Infected cells will then be tested for recognition by HER-2/neu specific T cells. Once expression is confirmed *in vitro*, we will titer the vectors *in vivo* for the generation of an anti-neu response. To accomplish this, 2 or 3 female FVB/N mice will be infected on day 0 and again on day 14 with the each vaccinia construct. Two weeks later, spleens will be excised and lymphocytes isolated for *in vitro* stimulation with neu-expressing targets. Lymphocytes will be tested 5-7 days later for recognition of neu expressing targets relative to wildtype targets. Vectors demonstrating *in vitro* neu-specific T cell generation will be tested further for the protection against challenge with the NT5 mammary tumor. In addition, studies will be set up to determine if these vectors can prevent the development of the spontaneous mammary tumors.

e. Recommendations in relation to the Statement of Work.

We had initially expected to complete constructing of all of vaccinia containing HER-2/neu constructs by the end of year two. Due to difficulties in constructing all of the constructs, we have had a slight delay. However, we are close to completing all of these constructs at this time, some of which are already being testing in vivo for immune priming (see below).

3. Testing of vaccinia constructs *in vivo.*

a. Procedure.

We performed an initial study to test the potency of the initial vaccinia vectors we constructed. Five mice per group were vaccinated on day 0 with a single intraperitoneal injection of either: 1) 10^7 pfu of the HER-2/neu gene mixed with $2x10^7$ pfu wildtype vector; 2) 10^7 pfu HER-2/neu gene mixed with 10^7 pfu of the GM-CSF gene and 10^7 pfu of the wildtype vector; 3) 10^7 pfu HER-2/neu gene mixed with 10^7 pfu of the GM-CSF gene and 10^7 pfu of the B7.1 vector; 4) 10^7 pfu HER-2/neu gene mixed with 10^7 pfu of the GM-CSF gene and 10^7 pfu of the B7.2 vector; 5) 10^7 pfu of the control HA gene mixed with 10^7 pfu of the GM-CSF vector and 10^7 pfu of the wildtype vector. Mice were challenged 2 weeks after vaccination with $5x10^6$ (parental mice) and $5x10^4$ (transgenic mice) NT5 mammary tumor cells subcutaneously in the right flank. Mice were monitored twice a week for the development of tumors.

b. Results.

As demonstrated in **figure 3**, we did not observe significant protection with the vaccinia constructs. However, we did observe that many of the mice in each group became ill.

c/d. Discussion and Future Studies.

We believe that mixing the recombinant vaccinia vectors with wildtype vector to standardize the concentration of total vaccinia vector given per mouse can explain the illness of the mice and the poor outcome of this study. The recombinant vectors are more attenuated than the wildtype vector and therefore require a higher concentration for immunization and result in less toxicity than the wildtype vector. We are therefore performing a series of titering experiments with each recombinant vaccinia construct. We will also dilute each construct with the attenuated control HA vector rather than wildtype vector in future studies to prevent toxcity.

e. Recommendations in relation to the Statement of Work.

We have completed the initial testing of vaccinia constructs in year 2 as planned. We expect to complete optimization of the vaccinia approach as well as to compare this approach with the previously tested approaches discussed above, during year 3 of this grant.

4. Evaluate recombinant vaccines for synergy with ablation of the mammary ductal system in preventing breast cancer.

We have developed a method for locally ablating the ductal epithelium by injecting via the mammary nipple, recombinant vectors carrying the thymidine kinase gene, followed by systemic administration of gancyclovir. We will eventually test whether the combination of this chemical ablation of mammary ductal epithelium with what is identified in the first two specific aims to be the best antigen-specific vaccine, can amplify the HER2/neu T cell response generated, thereby resulting in enhanced long-term prevention of mammary tumors in HER2/neu transgenic mice. During year one, we evaluated methods for mammary duct ablation with a vaccinia vector. During year 2, we tested this approach in an initial study together with the DNA plasmid vaccine.

a. Procedures.

Three mice per group were received either: 1) 1 ug of the HER-2/neu gene plus 1 ug of wildtype vector (pneu+pcDNA3); 2) 1 ug of the HER-2/neu gene plus 1 ug of the GM-CSF gene (pneu+pmGM-CSF); 3) 1 ug of the control HA gene plus 1 ug of the GM-CSF gene (pHA+pmGM-CSF); 4) 1 ug of the HER-2/neu gene plus 1 ug of wildtype vector followed by ductal ablation 2 weeks later (pneu+pcDNA3 (abl)); 5) 1 ug of the HER-2/neu gene plus 1 ug of the GM-CSF gene followed by ductal ablation 2 weeks later (pneu+pmGM-CSF (abl)); 6) 1 ug of the control HA gene plus 1 ug of the GM-CSF gene followed by ductal ablation 2 weeks later (pHA+pmGM-CSF (abl)); 7) no vaccine. Mice were monitored twice a week for the development of endogenous mammary tumors.

b. Results.

Mammary tumors developed in all treated mice. However, there was a delay in tumor development in mice treated with the pneu vectors with or without ablation.

c. Discussion.

This initial study only looked at a small group of mice. Because tumors develop over a three month period, we will need to evaluate a larger cohort of mice in each group.

d. Future studies.

We are planning to repeat this study with a much larger cohort of mice in each group. We will consult a statistician to aid in determination of the cohort size that would result in a statistically significant outcome. In addition, we plan to evaluate other non-vaccinia mediated methods of ablation to determine if they are more efficient at generating ablation and local inflammation. Finally, we plan to test other systemic vaccines for synergy with local ablation treatment.

e. Recommendations in relation to the Statement of Work.

We have completed the initial pilot study as planned in year two. We will continue to optimize the intraductal ablation procedure and evaluate synergy with other systemic vaccines in year three.

7. CONCLUSIONS.

This proposal seeks to develop a vaccine strategy that can specifically generate an immune response to ductal cells (the normal cells from which the majority of breast cancers arise), to preneoplasias, and to incipient tumor cells, by targeting common antigens expressed by these cells, as an alternative therapy for preventing breast cancer development. We are employing the HER-2/neu transgenic mouse model of breast cancer to evaluate vaccine strategies developed in our laboratory. We are currently comparing two approaches, plasmid DNA vectors and vaccinia vectors. So far, our data would suggest that plasmid DNA vectors delivered by gene gun can augment antitumor immunity. However, this immunity is further augmented by vaccinia vector immunization. We are in the process of optimizing the vaccinia vector approach and testing it in the HER-2/neu mouse for prevention of spontaneous mammary tumors. During the next year, we should complete testing of the vaccinia approach and begin testing of the listeria monocytogenes approach. In addition, we are currently testing novel manipulations of the immune system such as anti-CTLA-4 antibody administration

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9. APPENDICES.

1. **Figure 1.** Gene Gun Delivery of Plasmid DNA Vaccines is Successful at Generating Systemic Antitumor Immunity Against Mammary Tumors. See page 20.

2. **Figure 2.** Gene Gun Delivery of plasmid DNA Vaccines Generates Long-term Immunity against Tumors Expressing HER-2/neu. See page 21.

3. **Figure 3.** Pilot Study to Evaluate the Ability of a Vaccinia Vector Carrying the HER-2/neu gene to Prime HER-2/neu Specific Immune Responses. See page 22.

4. **Figure 4.** Pilot Study to Evaluate the Effects of a Plasmid DNA Vaccine Combined with Local Ductal Ablation to Prevent Breast Cancer Development. See page 23.

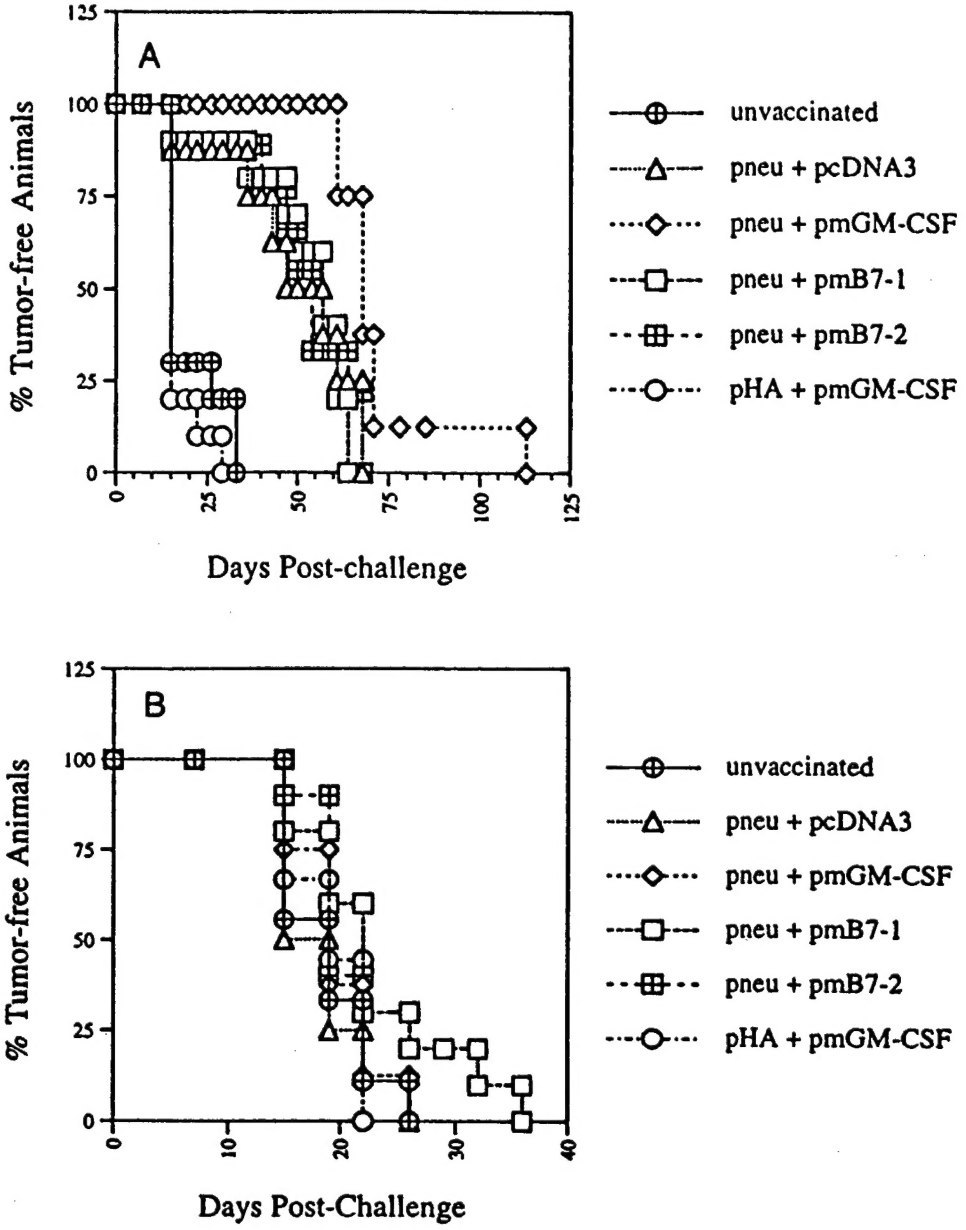

Figure 1. Gene Gun Delivery of Plasmid DNA Vaccines is Successful at Generating Systemic Antitumor Immunity Against Mammary Tumors.

A. 10 mice per group received intradermal injections of 1 ug of DNA in the left flank of either: 1) the HER-2/neu gene (neu) mixed with the pcDNA3 wildtype vector; 2) the HER-2/neu gene mixed with the GM-CSF gene (GM); 3) the HER-2/neu gene mixed with the B7-1 gene; 4) the HER-2/neu gene mixed with the B7-2 gene; 5) the hemagglutin (HA) control gene mixed with the GM-CSF gene as a negative control; 6) no vaccination. Mice were challenged 2 weeks after the last vaccination with $5x10^6$ (parental mice) and $5x10^4$ (transgenic mice) NT5 mammary tumor cells in the right flank. Mice were observed twice a week for the development of tumors. **A.** FVB/N mice. **B.** Transgenic Mice.

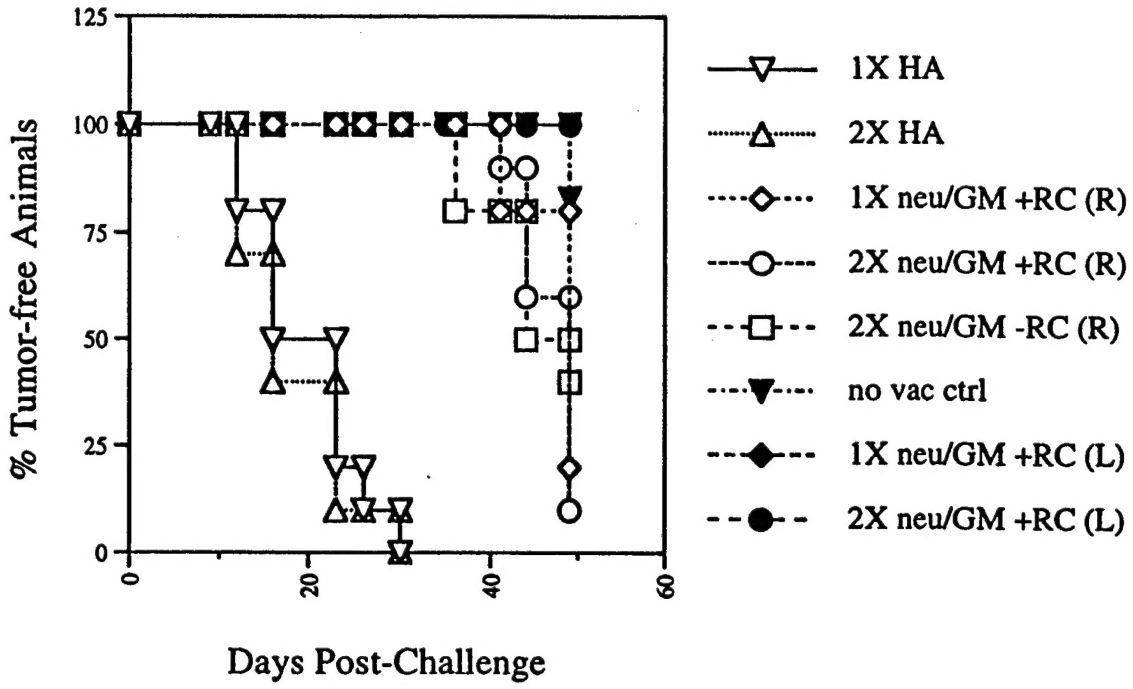

Figure 2. **Gene Gun Delivery of plasmid DNA Vaccines Generates Long-term Immunity against Tumors Expressing HER-2/neu.** FVB/N mice were vaccinated with DNA plasmids as described for experiment 1. Mice received either a single vaccination (1X) or two vaccinations (2X), each one week apart. Mice were challenged in the right flank (R) two weeks after the last vaccination with $5x10^6$ NT5 tumor cells. Mice were rechallenged in the left flank (L) on day 35 after the first challenge with fresh HER-2/neu expressing $5x10^6$ NT5 tumor cells. All mice were observed twice a week for the development of tumors. RC=rechallenge.

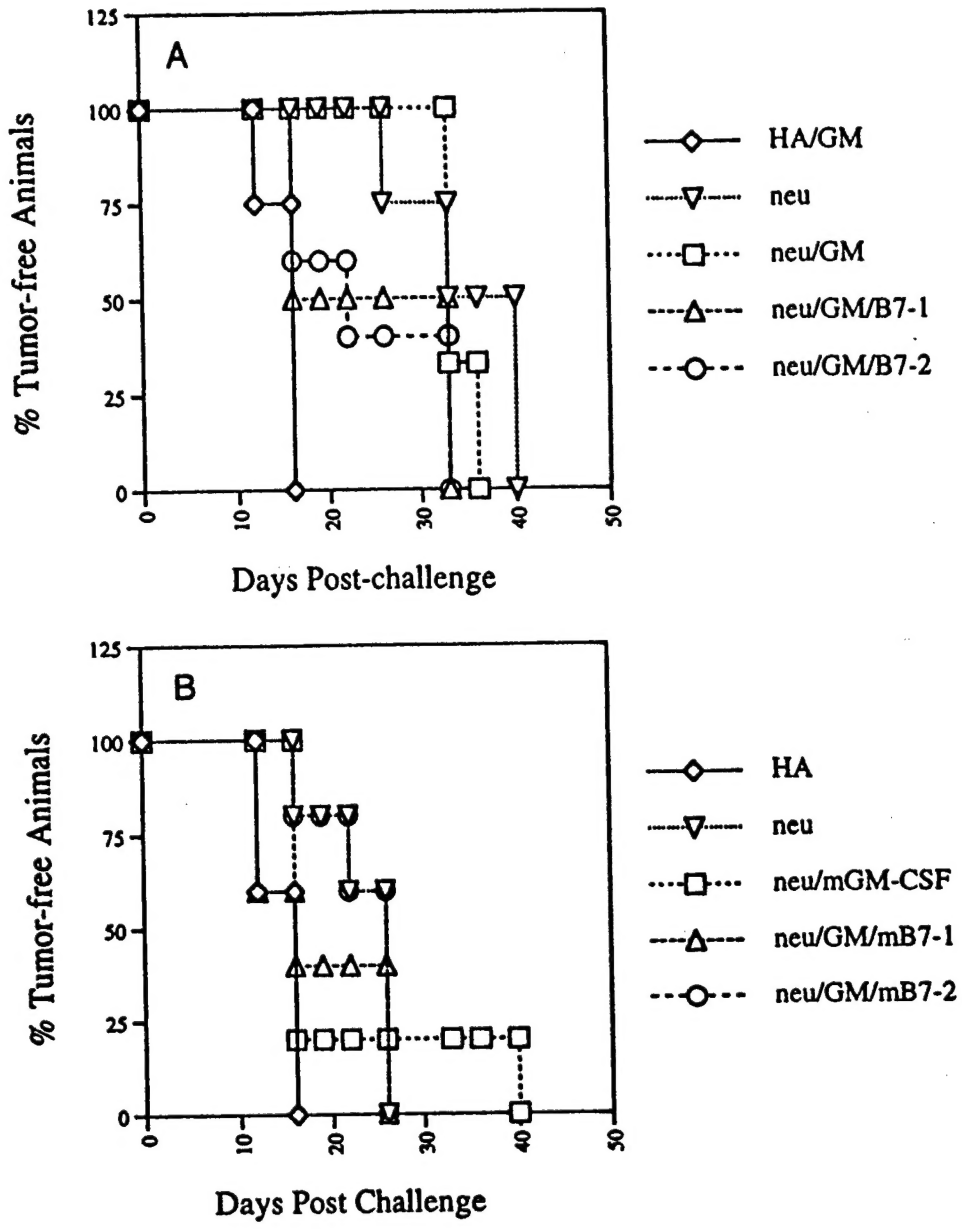

Figure 3. Pilot Study to Evaluate the Ability of a Vaccinia Vector Carrying the HER-2/neu gene to Prime HER-2/neu Specific Immune Responses. Five mice per group were vaccinated on day 0 with a single intraperitoneal injection of either: 1) 10^7 pfu of the HER-2/neu gene mixed with $2x10^7$ pfu wildtype vector (neu); 2) 10^7 pfu HER-2/neu gene mixed with 10^7 pfu of the GM-CSF gene and 10^7 pfu of the wildtype vector (neu/GM); 3) 10^7 pfu HER-2/neu gene mixed with 10^7 pfu of the GM-CSF gene and 10^7 pfu of the B7.1 vector (neu/GM/B7-1); 4) 10^7 pfu HER-2/neu gene mixed with 10^7 pfu of the GM-CSF gene and 10^7 pfu of the B7.2 vector (neu/GM/B7-2); 5) 10^7 pfu of the control HA gene mixed with 10^7 pfu of the GM vector and 10^7 pfu of the wildtype vector (HA/GM). Mice were challenged 2 weeks after vaccination with $5x10^6$ (parental mice) and $5x10^4$ (transgenic mice) NT5 mammary tumor cells subcutaneously in the right flank. Mice were monitored twice a week for the development of tumors. **A.** FVB/N mice. **B.** Transgenic mice.

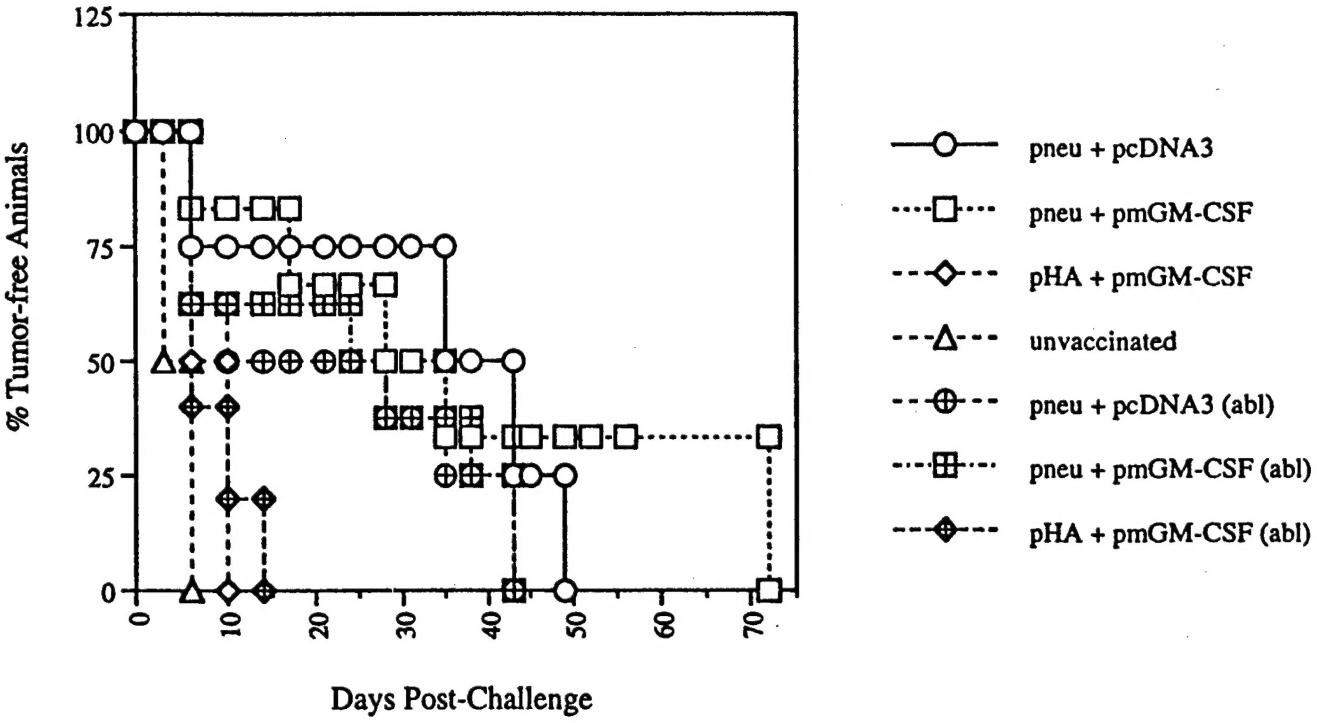

Figure 4. Pilot Study to Evaluate the Effects of a Plasmid DNA Vaccine Combined with Local Ductal Ablation to Prevent Breast Cancer Development.
Three mice per group were received either: 1) 1 ug of the HER-2/neu gene plus 1 ug of wildtype vector (pneu+pcDNA3); 2) 1 ug of the HER-2/neu gene plus 1 ug of the GM-CSF gene (pneu+pmGM-CSF); 3) 1 ug of the control HA gene plus 1 ug of the GM-CSF gene (pHA+pmGM-CSF); 4) 1 ug of the HER-2/neu gene plus 1 ug of wildtype vector followed by ductal ablation 2 weeks later (pneu+pcDNA3 (abl)); 5) 1 ug of the HER-2/neu gene plus 1 ug of the GM-CSF gene followed by ductal ablation 2 weeks later (pneu+pmGM-CSF (abl)); 6) 1 ug of the control HA gene plus 1 ug of the GM-CSF gene followed by ductal ablation 2 weeks later (pHA+pmGM-CSF (abl)); 7) no vaccine. Mice were monitored twice a week for the development of endogenous mammary tumors. Plotted are the mean time to development of tumor for the three mice in each group.